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Flow cytometric measurement of intracellular cytokines

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Abstract

The identification of distinct T helper lymphocyte subsets (Th1/2) with polarised cytokine production has opened up new fields in immunobiology. Of the several alternative methods of monitoring cytokine production, flow cytometric analysis of intracellular staining has distinct advantages and pitfalls. It allows high throughput of samples and multiparameter characterisation of cytokine production on a single cell basis without the need for prolonged in vitro culture and cloning. However, these methods may cause important changes in cell surface phenotype which can make interpretation difficult.
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Keywords: Cytokine; Flow cytometry; Intracellular staining

1. Background

Throughout the field of immunology, flow cytometry remains a defining technology. While some surface markers correlate with function, inferring function from surface staining remains an inexact art. Cells with similar surface phenotype may synthesise different products and have different functional characteristics.

The identification of functional subsets of $CD4^+$ T lymphocytes with polarised cytokine production was based originally on the characterisation of T cell clones using cytokine ELISA of culture supernatants (Mosmann et al., 1986). This method is impractical when faced with large numbers of heterogeneous cells obtained ex vivo. Making enough clones is highly laborious, and only a minority of effectors have clonogenic potential (Lalvani et al., 1997).

Several methods have been developed that allow cytokine expression to be measured: ELISA, ELISPOT, RT-PCR, LDA, ISH, immunohistochemistry and intracellular cytokine staining (ICCS). All have advantages and drawbacks: LDA, ELISPOT and ICCS are the most appropriate ways to estimate the frequency of cytokine producing cells; ELISA measures integrated amounts of secreted protein; RT-PCR measures semi-quantitative levels of inducible mRNA, while ISH and immunohistochemistry are useful for localisation of cytokine producing cells in tissues (see Table 1).

Intracellular cytokine staining was pioneered by

Abbreviations: APC, antigen-presenting cell; BAL, bronchoalveolar lavage; BSA, bovine serum albumin; DC, dendritic cell; DMSO, dimethylsulphoxide; ELISA, enzyme-liked immunoassay; ELISPOT, ELISA-based assay for detecting cells secreting analyte; FCS, fetal calf serum; ICCS, intracellular cytokine staining; ISH, in situ hybridisation; LDA, limiting dilution analysis; RT-PCR, reverse-transcription followed by polymerase chain reaction; TCR, T cell receptor; *S/N*, signal to noise ratio

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Table 1
Technologies for cytokine detection

	Intracellular cytokine stain	ELISPOT	ELISA	RT-PCR	Limiting dilution	In situ hybridisation	Immuno-histochemistry	Immuno-cytochemistry	RNA protection assay
Analyte	Protein	Protein	Protein	mRNA	Protein	mRNA	Protein	Protein	mRNA
Readout	Frequency	Frequency	Integrated amount	Integrated amount	Frequency	Localisation	Localisation	Localisation	Integrated amount
Equipment	Flow cytometer	Microscope or plate reader	Spectrophotometer	Thermal cycler	Spectrophotometer	Light/electron microscopy	Light/electron microscopy	Confocal microscope or EM	Gel set-up, phosphorimager
Cytokine co-expression	Yes	Yes (difficult)	No	No	Yes	Yes	Yes	Yes	No
Cell surface phenotype	Possible	Pre-selection	Pre-selection	Pre-selection	Possible	Possible	Possible	Possible	Pre-selection

the Anderssons in Stockholm in the 1980s, initially to immunostain tissue sections (Sander et al., 1991). After the introduction of methods to fix and permeabilise lymphocytes, the next development was the use of secretion inhibitors to accumulate cytokines intracellularly, allowing improvement of the signal/noise ratio (Schmitz et al., 1993; Jung et al., 1993). Finally, screening of large panels of monoclonal antibodies to select those that bind cytokines in their fixed form allowed practical methods to be developed (Openshaw et al., 1995).

Performing flow cytometric analysis on ICCS cells allows individual characterisation of large numbers of cells and can fully display the heterogeneity of cell populations. Compared to ELISA, a great advantage of ICCS is that multicolour staining can demonstrate exclusive or mutual co-expression of different cytokines in individual cells, thus allowing the characterisation of T cell subsets on the basis of cytokine production rather than just surface markers. Wide access to user-friendly flow cytometers has been another key element in the rapid and widespread adoption of ICCS in basic and clinical research.

In this review, we present our standard methods of flow cytometric analysis of intracellular cytokines and discuss current status, advantages and limitations of the technology.

2. Materials and methods

2.1. Basic materials

- PMA (phorbol 12-myristate 13-acetate Sigma P-8139)
- Ionomycin (Sigma I-0634)
- Calcium ionophore A23187 (500 ng/ml, Sigma C-9275)
- Brefeldin A (Sigma B-7651)
- Monensin (Sigma M-5273)
- Formaldehyde (Analar grade, 37–40%)
- Saponin (Sigma S-7900)
- DMSO (dimethyl-sulfoxide Sigma D-5879)
- Recombinant cytokines (Pharmingen and various other suppliers)
- FCS or BSA (Sigma A-7906)
- DNAse I

2.2. Stock reagents and buffers

- 'PMA and ionomycin' stock is made up in DMSO at 10 and 100 μg/ml, respectively, stored at −80°C. Thawed aliquots must not be re-used.
- Brefeldin A is dissolved in ethanol, 1 mg/ml, stored at −80°C in 100-μl aliquots.
- Monensin is dissolved in methanol by gentle warming at 37°C in a water bath. Stock is made up to 400 μM and stored at 4°C.
- PBS/0.1%NaN_3 (PN)
- PBS/1% FCS/0.1%NaN_3 (PFN) PBS/1% FCS/0.1%NaN_3/0.1% saponin (PFNS) (n.b.: FCS can be replaced with 1% BSA in all these buffers, particularly if biotin-free medium is required).
- PBS/S-milk: 5% (w/v) solution of non-fat dry milk in PBS. Mix with magnetic stirrer for 15 min and centrifuge at 1500×*g* for 30 min. Use supernatant, which should be clear or slightly opalescent. Store at 4°C.
- 4% formaldehyde in hypertonic PBS: 1.4 ml 10× PBS, 7.6 ml H_2O, 1.0 ml formaldehyde 37–40%.

- DNAse I 3 mg/ml in PBS (60000 Dornase U/mg). Store at −20°C.

2.3. Antibodies

Formaldehyde fixation and saponin permeabilisation are compatible with both indirect and direct staining, but the latter usually produces lower backgrounds, is simpler and faster and co-expression of different cytokines is more easily demonstrated. An expanding list of monoclonal antibodies suitable for ICCS in man, mouse and rat can be purchased from various suppliers. Typically PE- and FITC-conjugated antibodies are available, occasionally also APC and biotin conjugates. If possible, PE-conjugated antibodies should be reserved for the weakest signals, such as IL-4. Commercial sources include Pharmingen (www.pharmingen.com), R&D Systems (www.rndsystems.com), Sigma (www.sigma-aldrich-.com) and Medprobe Biosource International (www.Medprobe.com). Other manufacturers also have appropriate reagents. Isotype-matched controls and unlabelled anti-cytokine antibodies are also available.

- Mouse anti-human IFN-γ clone 4SB3 (IgG1), FITC-labelled, Pharmingen 18904A
- Mouse anti-human IL-4 clone 8D4-8 (IgG1), PE labelled, Pharmingen 18655A
- Rat anti-mouse/human IL-5 TRFK-5 (IgG1), PE labelled, Pharmingen 18055A
- Mouse anti-human CD4 clone Q4120 (IgG1), Quantum Red labelled, Sigma R-8886
- Isotype control MOPC21 (mouse IgG1) from Sigma, FITC-labelled (F6397), PE labelled (P-4685) and Quantum Red labelled (R-2138)
- Rat anti-mouse CD4 clone H129.19 (IgG2a) Quantum Red labelled, Sigma R3637
- Rat anti-mouse IL-10 clone JES5-2A5 (IgG2a) FITC-labelled, Pharmingen
- Rat anti-mouse IFN-γ clone AN18, PE labelled, DNAX
- Rat anti-mouse IL-4 clone 11B11 (IgG1), Pharmingen 18191A
- Rat anti-mouse IL-5 clone TRFK-5 (IgG1), Pharmingen 18051A (purified) and 18055A (PE labelled)
- Rat anti-mouse IL-2 clone JES6-5H4 (IgG2b), Pharmingen 18171A
- Rabbit anti-rat Ig, FITC labelled, Vector Laboratories cat. no. FI4001

2.4. Cells

Cytokines are produced by several cell types, including lymphocytes, basophils (Kon et al., 1998), eosinophils (Rumbley et al., 1999), antigen presenting cells such as dendritic cells (DC) (Kelleher and Knight, 1998), fibroblasts, but the majority of studies using ICCS so far have involved lymphocytes. Peripheral blood mononuclear cells (PBMC) are commonly used after separation on density gradients, but convenient whole blood methods have also been described (Ferry et al., 1997; Jason and Larned, 1997; Maino and Picker, 1998). Flow cytometry allows analysis of lymphocytes in complex mixtures such as bronchoalveolar lavage cells (BAL) without previous separation or in vitro culture. One disadvantage is the loss of histological information when exfoliated cells are analysed.

2.5. Differences between mouse and human ICCS

Techniques are broadly similar in working with cells form different species. However, saponin is commonly used at 0.5% for mouse cells and 0.1% for human cells. An important difference is that PMA and ionomycin stimulation causes less downregulation of CD4 in mouse lymphocytes compared to man.

2.6. Cell activation

Unless one is looking at cells that are already activated and producing cytokines (e.g., ex vivo studies of PBMC in chronic infections such as HIV, HTLV-1 (Kubota et al., 1998), where a small fraction of T cells expresses IFN-γ) some form of activation is required. This may be a polyclonal stimulus such as PMA and ionophore or PHA or anti-CD3 and anti-CD28 or, if the specificity is known, antigen presented by APC. The timing of this stimulus in relation to recent other stimuli is important, as T cells enter a refractory phase following stimulation. Thus T cell lines and clones maintained in culture are most responsive when their periodic re-stimulation is due (typically at 7–10-day intervals). The use of pharmacological activators or polyclonal ac-

tivators is often the only practical option with polyclonal responses, but the investigator should ascertain whether this induces the same cytokine profile as the physiological stimulus. For instance, PMA and ionomycin may induce a broader spectrum of cytokine production in human T cell clones than specific antigen presented by APC.

2.7. Method

A single cell suspension (PBMC, whole blood, BAL, mouse splenocytes, lymph node cells, thymocytes, etc.) is prepared in RPMI 1640 with 10% serum and usual supplements at $1–10\times10^6$ cells/ml and incubated at 37°C. PMA (10–50 ng/ml) and ionophore (ionomycin or A23187 at 100–500 ng/ml) are added and the suspension is vortexed. This is a very powerful activating stimulus acting on protein kinase C (PKC) and calcium ion influx, and is used both to induce cytokine expression of cells previously activated by physiological stimuli and as a positive control to show the potential expression by cells that respond weakly to a parallel stimulation with other stimuli, usually specific antigen. An incubation time of 4–6 h is adequate for most cytokines, but needs to be assessed for individual systems. Some cell death occurs with PMA and ionomycin, increasing with extensive incubations (>24 h). Significant loss of the most activated cells can affect the analysis, so the extent of cell death must be monitored. Also, dead cells release DNA strands, trapping other cells and forming clumps that interfere with further analysis. Adding DNAse (1:25, i.e., 2300 U/ml, with a 5-min incubation at 37°C) helps avoiding clumps. Block of cytokine secretion and intracellular accumulation is achieved by treatment with brefeldin A (10 μg/ml) or monensin (2–10 μM) (Dinter and Berger, 1998). Commercial alternatives exist, e.g., GolgiPlug (containing brefeldin A) and GolgiStop (containing monensin) both from Pharmingen. Secretion inhibitors can be added at the same time as the stimulus, or during the last 4–6 h of stimulation if using longer stimulation periods. Longer incubations with monensin (>12 h) are toxic to cells.

Alternatively, PHA (1–10 μg/ml) or superantigens (e.g., SEB at 2 μg/ml (Lee et al., 1990)) can be used on T cells, but APCs are required and particular care must be taken to disrupt clumps before fixation, to prevent artefacts and blockages during flow cytometric analysis. T cells can also be activated by TCR crosslinking by treatment with plate-bound anti-CD3 (1–10 μg/ml) and soluble anti-CD28 (1–10 μg/ml) for 24 h; optionally, cells can be expanded in the presence of IL-2 and IL-4 for 3 days and then stimulated with PMA and ionophore as above. According to the objective of the experiment, it may be desirable to minimise the extent and duration of in vitro manipulation.

If the specificity of the T cells is known, as with T cell lines and clones, antigen and APC can be added to achieve conditions that cause the cells to proliferate. Co-incubation of responder T cells and APC loaded with antigen must allow good cell–cell contact. This is best accomplished in round or conical based tubes or plates. A brief centrifugation ($400\times g$ for 5 min) helps, but prolonged centrifugation can lead to non-specific activation.

2.8. Fixation and permeabilisation

Formaldehyde fixation often best preserves cytokine antigenicity and scatter characteristics of cells without causing too great an increase in autofluorescence. We use 2% formaldehyde in hypertonic PBS. Permeabilisation is achieved by treatment with saponin (0.1–0.5%) in PBS/BSA. Ready made commercial mixtures exist which fix and permeabilise at the same time (e.g., Ortho Permeafix (Ortho), Cytofix/Cytoperm (Pharmingen)). It may sometimes be desirable to fix and preserve fixed cells for some time before permeabilisation and staining, for instance when running large experiments or time courses. For this reason we prefer to use separate fixation and permeabilisation.

Activated cells are washed once in PBS and resupended in 1 ml PBS by vortexing. While vortexing, an equal volume of 4% formalin in hypertonic (1.4×) PBS is added. Vortexing prevents clumping and must be maintained during the first minute of fixation. The cells are left in fixative for 20 min at room temperature, then washed once in PBS/BSA/azide for 10 min and resuspended in the same buffer for storage (4°C in the dark, up to 3 days) or directly processed for permeabilisation.

2.9. Surface markers

Classical T cell surface phenotype markers such as CD3, CD4 and CD8 and new markers such as the Th2-associated molecule T1/ST2 (Lohning et al., 1999; Xu et al., 1998) correlate to various degrees with cytokine production. Surface staining is best performed before fixation, as epitopes in surface markers may be destroyed by fixation and permeabilisation. However, it is not always possible to characterise stimulated T cells in terms of surface phenotype. Some markers are downregulated by the activation stimulus used to induce cytokine secretion. This limitation is particularly severe with CD4 in PMA and ionomycin-stimulated human T cells (Pelchen et al., 1993; Petersen et al., 1992; Ruegg et al., 1992), but other markers such as CD3, TCR (Telerman et al., 1987) and CD8 (Nakayama and Nakauchi, 1993) are also affected. This introduces a sort of 'uncertainty principle': it is possible to either determine accurately the surface phenotype or the intracellular cytokine production, but not both at the same time. Partial solutions include using intracellular staining for those markers (such as CD3) which are internalised but not degraded after PMA stimulation, or the use of TCR triggering, or using high concentrations of monensin to inhibit endosomal degradation. High concentration monensin may, however, inhibit cytokine production. For individual circumstances, a compromise has to be found. If using purified CD3-positive T cells, CD4 cells may better approximate to CD8-negative than CD4-positive cells (Meyaard et al., 1996), but $CD4^-$ $CD8^-$ $CD3^+$ cells are sometimes found, confounding this method. Alternatively, if activation does not require contact between different cell types (e.g., with PMA+ionomycin), cells can be pre-sorted on the basis of surface markers and then stimulated (Chipeta et al., 1998).

2.10. Positive controls

Activated, fixed cells can be obtained from commercial sources (e.g., Pharmingen). Alternatively PMA and ionomycin activation of T cell lines or clones with known cytokine production provides a convenient control. Such controls are mandatory in working up theses methods. The control cells should be as similar as possible to the cells that one intends to study.

2.11. Negative controls

Fixation increases autofluorescence and the non-specific trapping of antibodies. This causes an increase in fluorescence of fixed cells. In addition, permeabilisation increases the amount of protein available for non-specific interaction. Non-specific binding is best avoided by careful titration of staining antibody concentration (range is usually 0.5–5 μg/ml). Blocking the Fc receptor with IgG or anti-Fc antibodies may help. Inclusion of BSA, non-conjugated polyclonal immunoglobulin of the same species as the conjugated monoclonal, or other proteins in the staining buffer reduces the non-specific binding.

Isotype-matched controls are less useful than in surface staining. Their use requires demonstration that both anti-cytokine antibodies and isotype matched controls bind similarly to cytokine-negative fixed cells. A better specificity control is to block anti-cytokine antibody binding with a molar excess of pure cytokine. However, this can be expensive. An alternative is to incubate the cytokine-negative control cells with an excess of unlabelled anti-cytokine antibody, and the sample with an excess of isotype-matched control antibody. Then the same amount of labelled anti-cytokine antibody is added to both tubes (Prussin and Metcalfe, 1995; Prussin, 1997). Ultimately, a better demonstration of specificity is achieved by showing similar frequencies of positive cells by staining with different antibodies that bind to the same cytokine.

Cytokines such as IL-1β and low amounts of IFN-γ may be expressed both in surface bound and intracellular forms (Andersson et al., 1994; Assenmacher et al., 1996). This may complicate the setting up of the technique. Permeabilisation controls using directly labelled anti-vimentin or anti-β-actin antibodies may be useful.

2.12. Flow cytometry settings

Three- or four-colour cytofluorimetry for intracellular cytokine staining does not differ in principle from conventional surface staining. Appropriate elec-

tronic compensation is essential for multicolour staining, and may be problematic as the number of colours increases. As fixation/permeabilisation has a small but perceptible effect on scatter characteristics, backgating (checking the scatter characteristics of events gated on cytokine or surface marker fluorescence parameters) helps to confirm the identity of scatter defined cell populations. As with rare cell phenotypes, large numbers of events (>20 000–40 000) acquired in list mode (i.e., storing all acquired parameters for each individual cell) are useful for reliable determination of cytokines expressed at low frequency, such as IL-4 in normal PBMC. Ideally stimulation conditions have to be chosen to provide well separated bimodal distributions of fluorescence intensity in single-parameter histograms or distinct groupings in dot plots or contours plots. With the conventional region settings that allow 1% positive cells in the negative control sample, frequencies of positive cells below 1% cannot be evaluated.

3. Examples

3.1. Effect of different stimuli on the cytokine response of T cells

Resting cells do not normally make cytokines, so ICCS requires cells to be activated. Depending on the cytokine, levels peak some hours after stimulation then decrease within a few more hours or days (Openshaw et al., 1995). However, specific antigen, polyclonal mitogen and pharmacological activation may trigger different activation pathways. The investigator therefore needs to test whether a non-specific stimulus is equivalent to physiological activation for the purposes of the experiment.

To give an example, we wished to determine whether PMA+ionomycin would stimulate similar responses to antigen in a human T cell clone specific for house dust mite antigen Der p2. This clone was known to secrete IFN-γ, IL-4 and IL-5 when stimulated with APC and peptide Der p2 (21–40). Resting cells were therefore stimulated with PMA+ionomycin or antigen+APC. Brefeldin A was added and incubation stopped after 3, 4 or 5 h. Cells were fixed and permeabilised as described in the short protocol (Appendix A). IL-4 was detected using antibody 8D4-8-PE, IL-5 using antibody TRFK-5-PE and IFN-γ using antibody 4SB3-FITC.

Different patterns of cytokine production were obtained when cells were stimulated in different ways (Fig. 1). At 4 h, antigenic stimulation induced an IFN-γ response that was about one order of magnitude less than that induced by PMA+ionomycin, while IL-4 and IL-5 were about 28 and 44% of the non-specific response. Similar profiles were obtained at 3 or 5 h (data not shown).

3.2. Downregulation of CD4 following stimulation

The cell surface is a dynamic structure that changes in response to external or internal influences. Activation is accompanied by downregulation of integral membrane proteins such as the TCR/CD3 complex, CD4, CD8, and glycosylphosphatidylinositol (GPI) anchored molecules such as CD14, CD16, and cytokines produced through activation can in turn downregulate their own receptor or those for other cytokines. Downregulation by shedding, as in the case of GPI-linked proteins, is intractable. Downregulation by endocytosis may be followed by recycling to the cell surface, as in the case of CD3 and the TCR, or degradation in phagolysosomes, as in the case of CD4. Thus agents that inhibit the acidification of phagolysosome can allow detection of surface markers after they have been endocytosed.

We tested the effect of PMA+ionomycin on CD4 expression and IFN-γ induction in human $CD4^+$ T cell clone HA1.7, previously maintained in culture by weekly restimulation with specific antigen (HA peptide 306–318) and APC. Increasing doses of PMA 0, 0.1, 1, 10 and 50 ng/ml were tested, combined with monensin at 0, 2, 10 or 50 μM. The cells were stained with anti-CD4–Quantum Red at 4 h, fixed, permeabilised and stained with anti-IFN-γ. Using a scatter gate for lymphocytes, percentages of cells positive for CD4 or IFN-γ were determined based on isotype matched controls.

In the absence of monensin, 2% of unstimulated cells expressed intracellular IFN-γ. This increased to 48% with 1–10 ng/ml PMA, decreasing again at 50 ng/ml. The expression of CD4 (89% of cells before stimulation) decreased progressively with increasing

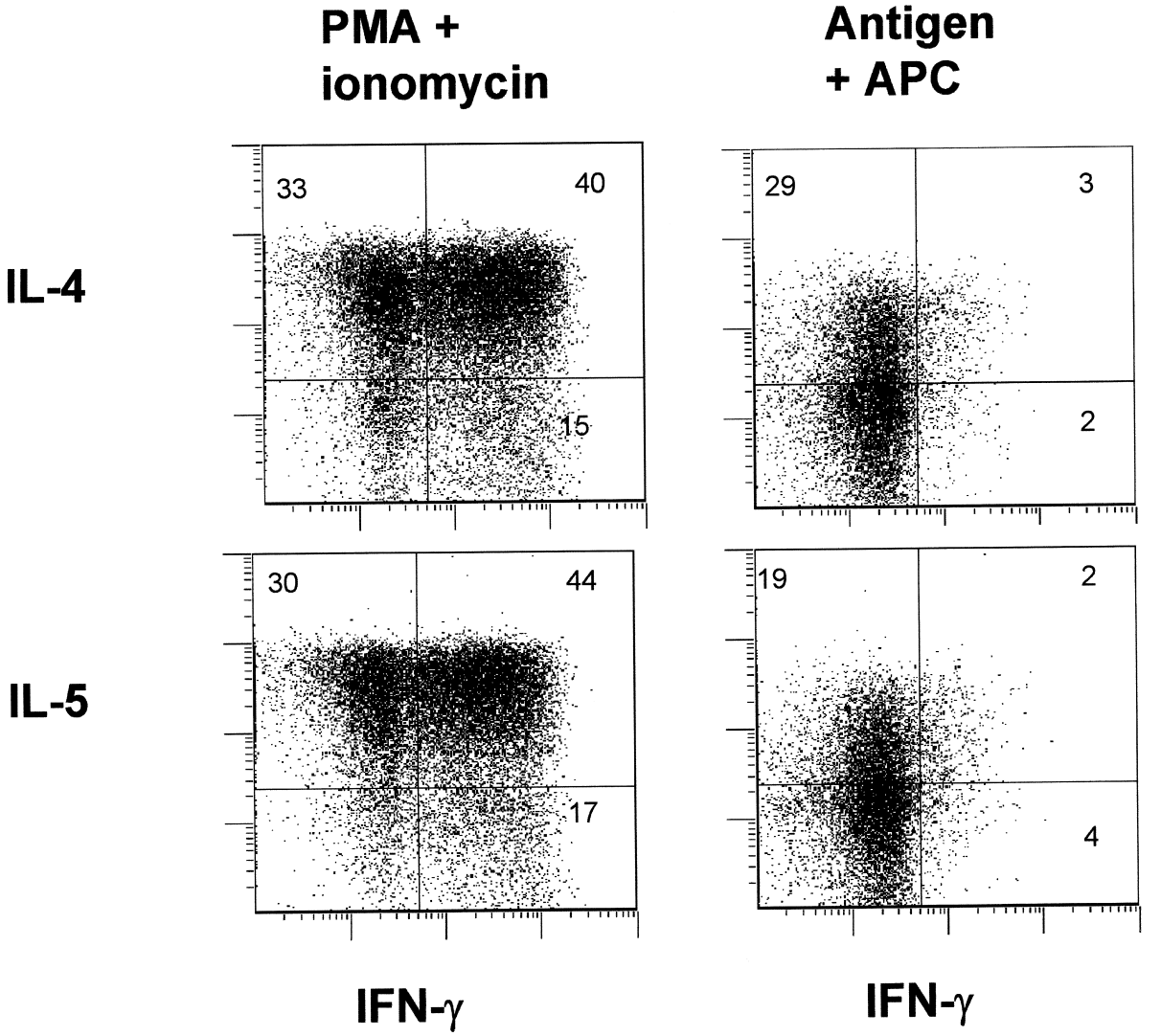

Fig. 1. Different patterns of cytokine expression in a human T cell clone after stimulation with mitogen or specific antigen. Human T cell clone AC1.1 was stimulated with PMA and ionomycin or antigen and APC for 4 h in the presence of 2 μM monensin. The cells were then fixed, permeabilised and stained with anti-IL-4 and or with anti-IL-5 and anti-IFN-γ. Numbers inside quadrants represent percentages of gated lymphocytes.

doses of PMA (Fig. 2a). Inclusion of monensin limited the downregulation of CD4 and increased the proportion of cells scoring positive for IFN-γ expression, but at the highest doses of monensin (50 μM), IFN-γ expression was also impaired (Fig. 2b–d).

Although accurate determination of both CD4 and IFN-γ expression in a single cell is technically possible, activation can have profound effects on both intracellular cytokine expression and display of surface markers.

3.3. Activation by antigen or PMA and ionomycin causes CD4 downregulation

There are multiple, partially overlapping and incompletely defined pathways of T cell activation.

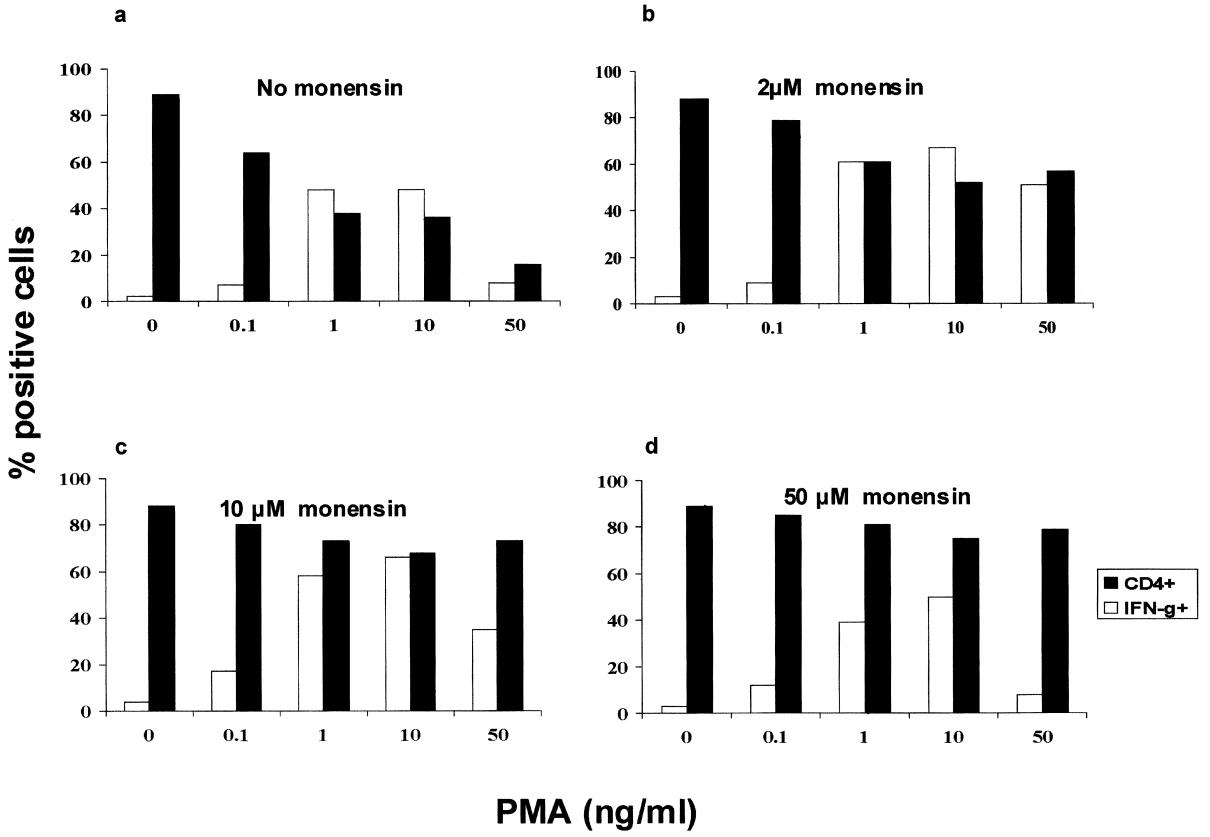

Fig. 2. Effect of monensin on mitogen-induced IFN-γ expression and downregulation of CD4. Human $CD4^+$ T cell clone HA1.7 was stimulated with various concentrations of PMA and 500 ng/ml ionomycin for 4 h in the presence of 0, 2, 10 or 50 μM monensin. The cells were then stained with anti-CD4, fixed, permeabilised and stained with anti-IFN-γ.

We wished to determine whether CD4 downregulation was a feature of artificial activation with PMA and ionomycin or whether it also occurred with physiological activation by antigen presented by antigen presenting cells.

Human $CD4^+$ T cell clone AC1.1 was therefore stimulated with PMA and ionomycin or by co-incubation with HLA-$DR11^+$ PBMC and specific antigen (5 μg/ml of Der p 2 peptide 28–40). Cultures received 10 μg/ml brefeldin A during the last 2 h of incubation. Samples were fixed at 0, 4 and 6 h, permeabilised and stained with anti-CD4–Quantum Red and anti-IL-4–PE.

Flow cytometric analysis showed that 99% of resting cells in a scatter gate for lymphocytes were positive for CD4 and negative for IL-4 at time 0 for the clone stimulated with PMA and ionomycin. Contaminating APC present in the same gate lowered the $CD4^+$ to 88% for the cultures stimulated with peptide. Following either stimulus, a large fraction of cells expressed IL-4 at 4 and 6 h. This was accompanied by a considerable downregulation of CD4: by 6 h only 13.2% (or 6.8%) of the cells stimulated with PMA+ionomycin (or antigen) remained $CD4^+$ (Fig. 3). Both specific and non-specific stimulation therefore induce CD4 downregulation.

We find CD4 downregulation following PMA and ionomycin activation not to be a problem with mouse lymphocytes. For example, we stimulated resting normal BALB/c splenocytes with PMA and ionomycin for 4 h, adding brefeldin A during the last 2 h of incubation. Untreated resting cells and cells infected with RSV (1 pfu/cell) overnight before

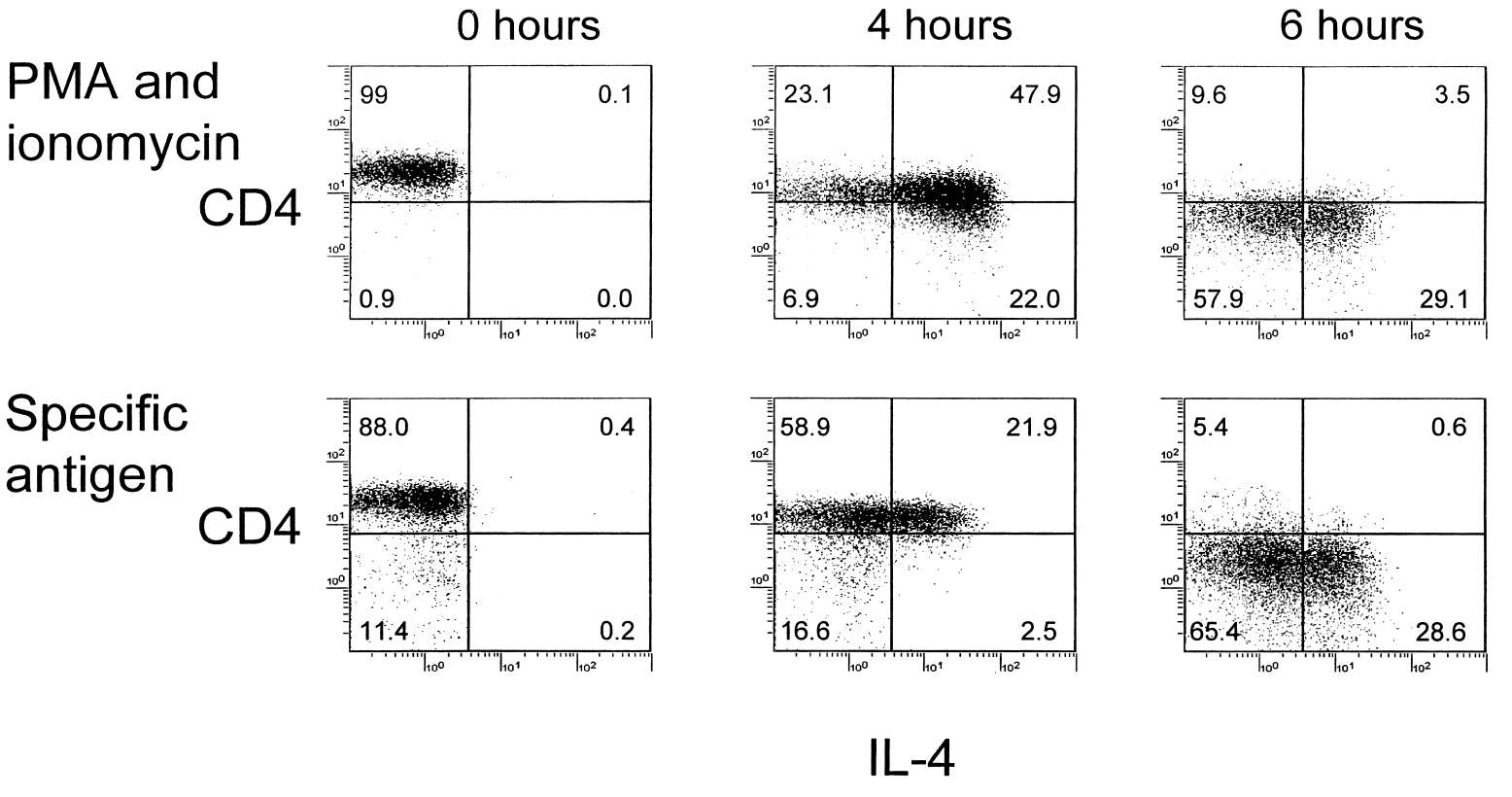

Fig. 3. Downregulation of CD4 following mitogenic or antigenic stimulation. Human T cell clone AC1.1 was stimulated with PMA and ionomycin or specific antigen. Incubations lasted 0, 4 or 6 h. Brefeldin A was present during the last 2 h of incubation. The cells were then stained with anti-CD4, fixed, permeabilised and stained with anti-IL-4. The dot plots show the co-expression of CD4 and IL-4. Numbers inside quadrants represent percentages of gated lymphocytes.

stimulation were also examined. Although stimulation with PMA and ionomycin or RSV infection decreased the mean CD4 fluorescence intensity, $CD4^+$ lymphocytes remained easy to distinguish, with a minimal decrease in the percentage of positive cells (Fig. 4).

3.4. Co-expression of IFN-γ and IL-10 in murine $CD4^+$ T cells

One major advantage of intracellular staining and flow cytometric analysis is the ability to study multiple markers and cytokines simultaneously on individual cells. To look at the ability of cells to make two or more cytokines simultaneously, we designed the following experiment. The experimental set-up is described in detail in our recently published studies (Hussell et al., 1996).

BALB/c mice were immunised by scarification with recombinant vaccinia expressing the attachment protein G and challenged with RSV (5×10^6 pfu) by intranasal inoculation. On day 7 after challenge, BAL cells were recovered (Spender et al., 1998). The cell suspension was stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4 h and cytokine secretion was blocked with brefeldin A (10 μg/ml) added during the last 2 h of incubation. The cells were then stained with anti-CD4–Quantum Red, followed by fixation, permeabilisation and staining with anti-IFN-γ and anti-IL-10. Co-expression of IL-10 and IFN-γ was then evaluated in lymphocytes gated for CD4 expression.

A large proportion of lung lymphocytes expressed IFN-γ on day 7 of infection. Some of the cells with the highest levels of IFN-γ expression also synthesised IL-10, showing that IL-10 is produced by a subset of IFN-γ producing cells (Th1), not by Th2 cells. Indeed, in the RSV model we do not see IL-10 without IFN-γ expression (Fig. 5).

3.5. Kinetics of expression of different cytokines in TH1 and TH2 long-term clones

To assess the relative kinetics of expression of IFN-γ, IL-2, IL-4, IL-5 and IL-10 in mouse T cells,

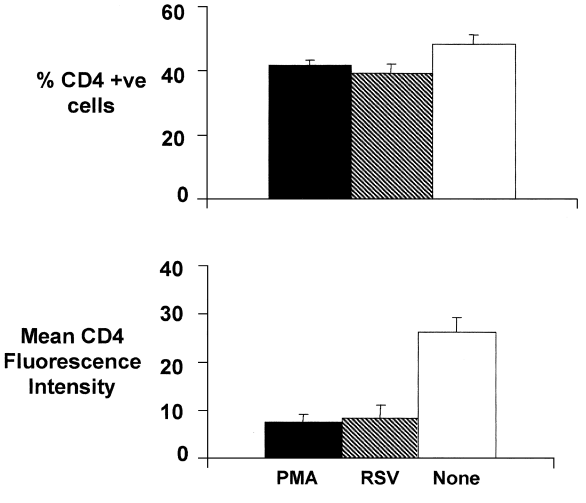

Fig. 4. Loss of CD4 fluorescence intensity in activated mouse T cell cultures. Naive BALB/c splenocytes were stimulated with PMA and ionomycin for 4 h, infected with RSV (1 pfu per cell) or incubated in medium alone overnight. Brefeldin A was included during the final 2 h. The cells were then stained with anti-CD4 and analysed for expression. Mean fluorescence intensity (MFI) is in arbitrary units.

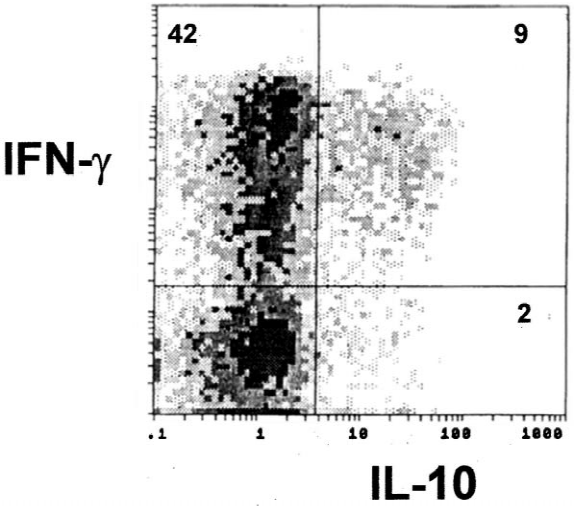

Fig. 5. Co-expression of IFN-γ and IL-10 in murine lung $CD4^+$ T cells. BALB/c mice were immunised with vaccinia virus expressing RSV attachment protein (G), then challenged intranasally by RSV infection. Bronchoalveolar lavage cells were recovered on day 7 of RSV infection, stimulated with PMA and ionomycin for 4 h with brefeldin A added during the last 2 h of incubation. After staining with anti-CD4–Quantum Red, the cells were fixed, permeabilised and stained with anti-IFN-γ –PE and anti-IL-10–FITC. Co-expression of IFN-γ and IL-10 is shown in lymphocytes gated for CD4 expression. Numbers inside quadrants represent percentages of gated lymphocytes.

long-term Th1 clone HDK-1 (KLH specific) and Th2 clone CDC25 (rabbit Ig-specific) were stimulated with PMA and ionomycin and cultured for up to 48 h, with brefeldin A added for the last 2 h. Whereas IFN-γ was synthesised by the majority of Th1 cells by 4 h and remained sustained for up to 48 h, production of IL-4 and IL-10 by Th2 cells peaked at 4 h (60% of cells positive for IL-10, 33% for IL-4) and decreased rapidly; IL-5 expression followed a slower kinetics, peaking between 8 and 16 h (Fig. 6). IL-2 was detected in a low percentage of cells (maximum 15%) of either clone at any time. These results illustrate a clear difference in the kinetic of expression of different cytokines.

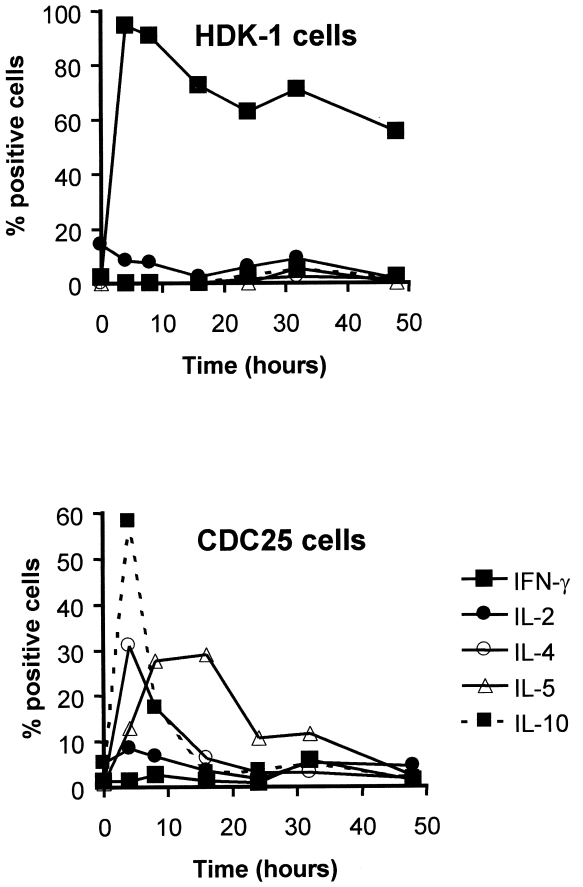

Fig. 6. Kinetics of intracellular expression of IFN-γ, IL-2, IL-4, IL-5 and IL-10 in mouse Th1 and Th2 clones. Mouse T cell clones HDK-1 (KLH-specific) and CDC25 (rabbit Ig-specific) were used 10–14 days after stimulation with antigen and irradiated spleen cells. The resting cells were washed and restimulated with PMA and ionomycin for various times, and brefeldin A was added for the last 2 h. Cells were fixed, permeabilised and stained for cytokines by incubation with anti-cytokine antibodies or isotype controls for 30 min, washed and incubated with FITC-conjugated rabbit anti-rat IgG for 30 min. After two washes, purified rat IgG (300 μg/ml) was added for 10 min to block residual anti-rat IgG binding, followed by anti IFN-γ-PE or control PE-conjugated rat IgG for 20 min, washed and analyzed. Percentages of positive cells were based on negative isotype-matched controls.

4. Discussion

The widespread use of flow cytometry has fundamentally affected the way we view the structure and lineage of the cells that comprise the immune system. This revolution, based on surface markers, is now being followed by further refinements based on staining for internal antigens. Among these, intracellular cytokine staining has played an important part in defining functional subsets in mixed T cell populations. It allows both the frequency of cells producing specific cytokines to be estimated and, to some extent, the levels of expression to be compared. It is also possible to investigate co-expression of different cytokines without the laborious and erratic pitfalls of cloning. The high throughput that flow cytometry can achieve is a major benefit in analysis of complex populations obtained ex vivo.

There are, however, a number of important pitfalls. Permeabilisation frequently causes high autofluorescence and many antibodies that bind specifically in other conditions do not work well on permeabilised, fixed cells. Some cytokines seem to be expressed at relatively low levels, and appear as a continuous shoulder on a histogram of fluorescence, instead of being a well-separated bi-modal distribution. For all these reasons, use of appropriate negative and positive controls is of paramount importance.

Cytokine synthesis is generally not constituitive, and only a small proportion of cells obtained ex vivo stain for intracellular cytokines. This is, perhaps, surprising given that cells are often obtained from sites of active inflammation. The explanation may lie in the kinetics of activation and in asynchronous cytokine production by individual cells. It is well established that even clonal populations of lymphocytes produce different cytokines asynchronously

(Openshaw et al., 1995). Different cytokines have different kinetics of expression, so that optimal times for detection vary. These factors limit investigation of co-expression of different cytokines in single cells. It may be possible to determine how many cells co-express two or more cytokines at a single point in time, but the sequential expression of cytokines by the same cells may be impossible to discover by this technique.

By comparison to some alternative methods, intracellular staining can be relatively insensitive, even after using monensin or brefeldin A to cause intracellular accumulation. ELISA for secreted cytokines measures the integrated accumulation of cytokine, balanced by consumption of cytokine in the culture. In addition, it must be remembered the presence of intracellular cytokine does not equate to secretion of that cytokine in vivo, or to its biological effects. ELISA has inherently greater specificity than intracellular staining, since most ELISAs use sandwich techniques in which two different epitopes of the cytokine must be recognised in order to provide a signal. For example, a single antibody recognising histone 2b and human IL-6 could give misleading results if used to stain for intracellular IL-6, but not if the antibody were one of a pair used for an ELISA (Zunino et al., 1996).

Intracellular cytokine staining has been used extensively to characterise immune responses in normal and diseased states (summarised in Table 2). In humans, most studies have relied on PBMC, although sinovial fluid, tissue infiltrating lymphocytes, eosinophils, basophils and dendritic cells have also been examined. Although PBMC are easier to obtain than locally infiltrating cells, it is important to recognise that relevant cells may accumulate in inflamed tissues and be depleted form the circulation during acute inflammatory conditions. The PBMC may actually contain those cells that are irrelevant to the response, having been left behind in the circulation or rejected by the tissues. The frequency of specific cells may therefore increase during convalescence (Isaacs et al., 1987; Isaacs et al., 1991).

Another important pitfall is to equate the number of cells that stain or intensity of staining with the importance of the cytokine. In our experience, Th2 cytokines may be hard to detect and present only transiently after stimulation in a minority of cells. By contrast, IFN-γ and TNF-α may be present in a very high proportion of cells for a long time after stimulation and stain very brightly. This does not, however, indicate that the biological outcome is dominated by the effects of say, IFN-γ. Our studies in the mouse have shown abundant IFN-γ producing cells are often present in situations in which lung eosinophilia occurs, although the eosinophilia is due to a switch from Th1 to Th2 and the production of IL-4 and IL-5. The frequency of IFN-γ producing cells is reduced in eosinophilic mice, but they remain the dominant cell population (Spender et al., 1998). Our interpretation is that IFN-γ has relatively weak effects locally and that this weakness is corrected for by its abundance. On the other hand, IL-4 and IL-5 are very potent and only need to be produced by very few cells in order to dominate the immunopathological process.

It is similarly wrong to think that an abundant cell type must be central to the pathogenesis of a response. In our cell transfer studies, the lung pathology that resulted from T cell transfers was dominated by an abundance of other cells that were recruited as part of the inflammatory process initiated by the transferred cells; the transferred cells were hard to discern amongst the bystanders (Cannon et al., 1988).

A further pitfall is to rely on the expression of surface markers that are downregulated following activation. For example, in examining IFN-γ in human PBMC, it is possible to lose much of the surface CD4 staining after stimulation in vitro. It might therefore be concluded that the production of IFN-γ is from $CD4^-$ cells, perhaps ascribing it to NK cells or CD8 cells. It is therefore important to determine the effect of stimulation protocols and fixation on the surface expression of cell lineage markers. This is an important potential source of artefacts.

A final major limitation of intracellular cytokine staining is that cells have to killed in order to visualise cytokines. Further functional studies are therefore precluded, allowing only a snapshot, static view of cytokine production to be obtained. Current developments include the use of very sensitive surface staining techniques which do not require fixation. The ingenious development of magnetofluorescent liposomes to detect surface IFN-γ (As-

Table 2
Synopsis of recent studies using intracellular cytokine staining

Condition		Cell type	Reference
Autoimmune diseases	Multiple sclerosis	PBMC T cells	Becher et al., 1999; Crucian et al., 1996
	Inflammatory bowel disease, SCID mouse LP infiltrating T cells	PBMC, IEL and lamina propria MC	Bregenholt and Claesson, 1998; Meenan et al., 1998; Simpson et al., 1997; Thoma et al., 1998
	Rheumatoid arthritis	PBMC, synovial fluid T cells	Kusaba et al., 1998; Morita et al., 1998
	Hashimoto or Graves thyroiditis	Thyroid infiltrating T cells	Roura et al., 1997
	Systemic lupus erythematosus	PBMC T cells	Garcia et al., 1997
	Behcet's disease	PBMC	Sugi et al., 1998
Neoplasia	Melanoma, gastric carcinoma	Tumour infiltrating lymphocytes PBMC T cells, LN	Labarriere et al., 1998; Sato et al., 1998a,b; Sommer et al., 1998a; Tabata et al., 1999; Hoyle et al., 1998
Transplantation	Kidney, mouse heart, haemodiafiltration	T cells from solid tissue, PBMC	Paglieroni et al., 1999; Panichi et al., 1998; Stinn et al., 1998
Immunodeficiency	Common variable immunodeficiency	PBMC	North et al., 1996; North et al., 1998
Atopy/allergy	Atopic dermatitis	Whole blood, PBMC T cells, basophils	Kon et al., 1998; Ferry et al., 1997; Jung et al., 1995; Nakagawa et al., 1998; Nakazawa et al., 1997; Sato et al., 1998a,b
	Asthma	Peripheral blood T cells	Krouwels et al., 1997; Krug et al., 1998; Krug et al., 1997; Randolph et al., 1999
Viral infections	HIV, SIV	T cells, monocytes, macaque IEL	Collins et al., 1998; Estcourt et al., 1997; Honda et al., 1998; Jason et al., 1999; Lecoeur et al., 1998; Meyaard et al., 1996; Sato et al., 1998a,b; Smit et al., 1998; Westby et al., 1998
	HTLV-I	PBMC	Kubota et al., 1998
	Herpes simplex virus	Cord blood and PBMC	Ito et al., 1998
	Epstein-Barr virus	PBMC	Nazaruk et al., 1998
	Influenza virus	T cells	Falchetti et al., 1998
	Respiratory syncytial virus	BAL, LN, Lung T cells	Hussell et al., 1998; Hussell and Openshaw, 1998; Openshaw et al., 1998; Murphy et al., 1996 Openshaw et al., 1995; Hussell and Openshaw, 1998; Hussell et al., 1996, 1997a,b
	Measles	PBMC	Ito et al., 1997
	Lymphocytic choriomeningitis virus	T cells	Murali et al., 1998; Oxenius et al., 1999; Su et al., 1998
Bacterial infections	Lyme arthritis	Synovial T cells	Gross et al., 1998
	Listeria monocytogenes	Peritoneal T cells	Kadena et al., 1997
	Bacillus Calmette Guerin (BCG)	PBMC	Sander et al., 1995
Parasitic infections	Schistosomiasis	Spleen T cells, eosinophils	Lohning et al., 1999; Fallon et al., 1998; Rumbley et al., 1999
	Leishmaniasis	T cells	Sommer et al., 1998b
	Bancroftian filariasis	PBMC	de Almeida et al., 1998
	Alveolar echinococcosis	PBMC	Jenne et al., 1998
Trauma	Burns	PBMC	James et al., 1996
Normal	Mouse	Dendritic cells, purified T cells, LN cells	Kelleher and Knight, 1998; Miner and Croft, 1998; Ulrich and Vohr, 1996
	Rat	T cell clones	Knudsen et al., 1997
	Man	Whole blood, cord blood, PBMC	Chalmers et al., 1998; O'Mahony et al., 1998; Annunziato et al., 1997;
		T cells monocytes, spleen slice culture,	Chipeta et al., 1998; Sewell et al., 1997; Hamann et al., 1996;
		T cell clones, milk mononuclear cells	James et al., 1996; Skibinski et al., 1997; Jung et al., 1996; Skansen et al., 1993

senmacher et al., 1996), and the isolation of single living cells inside microdroplets of a porous biomatrix that traps secreted products long enough to allow the detection using fluorochromed-tagged antibodies is another (Gift et al., 1996; Nir et al., 1990). Another novel and promising approach is the biotinylation of the surface of living cells, followed by incubation with avidin-conjugated antibodies. Secreted cytokine is then bound to the anticytokine antibody on the surface, allowing its detection by a second fluorochrome conjugated antibody (Manz et al., 1995). Such approaches are technically demanding, but are powerful additions to the techniques that allow us to follow the fate of differentially activated lymphocytes as they develop. These and other new methods may extend the applications of flow cytometry to measurement of cytokine production in the near future.

Appendix A. A short protocol for IFN-γ and IL-4 staining of human T cells

Basic materials and stock reagents and buffers are listed above. Additional requirements are: human PBMC, T cell lines or clones in the resting phase (when due for re-stimulation). N.B. IL-4-producing cells are very rare in normal PBMC. Th2 clones make better positive controls for IL-4 staining.

Procedure

Stimulation.

- Set up T cell cultures at 10^6/ml in RPMI 1640 complete culture medium in 15-ml tubes (or 24-well plates), 1–2 ml per tube.
- Add PMA+ionomycin to 10 and 100 ng/ml, respectively, and monensin to 10 μM.
- Treat non-stimulated controls with monensin alone.
- Incubate for 4 h at 37°C.

Surface staining.

- Add 10 ml PN to each tube, pellet cells, resuspend in PN.
- Aliquot cells into groups to be stained with anti-CD4 and its isotype control.
- Add anti-CD4-QR (0.15 μg/million cells), incubate 30 min at 4°C, wash in PN.

Fixation.

- Resuspend cells in 1 ml PN. Vortex.
- While still vortexing, add 1 ml formaldehyde 4% in hypertonic PBS.
- Incubate 20 min at room temperature.
- Add 10 ml PFN. Pellet cells and wash in PFN.
- Fixed cells can be kept 3 days in PFN at 4°C, protected from light.

Permeabilisation.

- Pellet cells ($1500\times g\times 10$ min, as fixed cells pellet poorly) and resuspend in 1 ml PFNS.
- Incubate 10 min at room temperature.

Intracellular staining.

- Set up antibody mixtures in PFNS. Purified 4SB3-FITC is used at 0.5 μg/10^6 cells, 8D4-8-PE at 0.25 μg/10^6 cells. Isotype controls are used at the same concentration as their matching antibody.
- Staining can be performed in tubes or in 96-well plates (V-bottom).
- For the following method, we assume 2×10^5 cells in 200 μl/well in a 96-well plate.
- Pellet cells by centrifuging the plate for 1 min at 2000 rpm.
- Remove *S*/*N* by quickly flicking the plate over a sink.
- Add 50 μl/well of anti-cytokine antibody mixture. Mix well.
- Incubate 30 min at room temperature in the dark, preferably on a plate shaker.
- Wash three times with 200 μl/well PFNS.

Flow cytometry.

- Run samples on flow cytometer after performing electronic compensation using single stained FITC, PE and Quantum Red samples.
- In list mode, acquire >40 000 events in a lymphocytes scatter gate.

- Set regions so that ~1% of unstimulated cells are positive for cytokines.
- Compare CD4 expression in stimulated and unstimulated lymphocytes.
- Compare IFN-γ and IL-4 in stimulated and unstimulated lymphocytes.
- Compare IFN-γ and IL-4 in CD4-positive and -negative gated lymphocytes.

Expected result.

- IFN-γ should be detectable in 15–30% adult PBMC, IL-4 in 1–2% at 4 h.
- >50% of clone AC1.1 will express IFN-γ or IL-4 at 4 h. CD4 downregulation should also be seen.
- IFN-γ-positive cells should appear as a distinct population in both PBMC and T cell clones. IL-4 may appear as a shoulder in a PBMC histogram, and a biphasic histogram for cloned T cells. The whole procedure can be performed in a day, although with larger experiments or time courses it is easier to collect and fix samples on day 1 and permeabilise, stain and analyze on day 2.

References

Andersson, J., Abrams, J., Bjork, L., Funa, K., Litton, M., Agren, K., Andersson, U., 1994. Concomitant in vivo production of 19 different cytokines in human tonsils. Immunology 83, 16.

Annunziato, F., Manetti, R., Cosmi, L., Galli, G., Heusser, C.H., Romagnani, S., Maggi, E., 1997. Opposite role for interleukin-4 and interferon-gamma on CD30 and lymphocyte activation gene-3 (LAG-3) expression by activated naive T cells. Eur. J. Immunol. 27, 2239.

Assenmacher, M., Scheffold, A., Schmitz, J., Checa, J.A.S., Miltenyi, S., Radbruch, A., 1996. Specific expression of surface interferon-gamma on interferon-gamma producing T cells from mouse and man. Eur. J. Immunol. 26, 263.

Becher, B., Giacomini, P.S., Pelletier, D., McCrea, E., Prat, A., Antel, J.P., 1999. Interferon-gamma secretion by peripheral blood T-cell subsets in multiple sclerosis: correlation with disease phase and interferon-beta therapy. Ann. Neurol. 45, 247.

Bregenholt, S., Claesson, M.H., 1998. Increased intracellular Th1 cytokines in scid mice with inflammatory bowel disease. Eur. J. Immunol 28, 379.

Cannon, M.J., Openshaw, P.J.M., Askonas, B.A., 1988. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. J. Exp. Med. 168, 1163.

Chalmers, I.M., Janossy, G., Contreras, M., Navarrete, C., 1998. Intracellular cytokine profile of cord and adult blood lymphocytes. Blood 92, 11.

Chipeta, J., Komada, Y., Zhang, X.L., Deguchi, T., Sugiyama, K., Azuma, E., Sakurai, M., 1998. $CD4^+$ and $CD8^+$ cell cytokine profiles in neonates, older children, and adults: increasing T helper type 1 and T cytotoxic type 1 cell populations with age. Cell Immunol. 183, 149.

Collins, D.P., Luebering, B.J., Shaut, D.M., 1998. T-lymphocyte functionality assessed by analysis of cytokine receptor expression, intracellular cytokine expression, and femtomolar detection of cytokine secretion by quantitative flow cytometry. Cytometry 33, 249.

Crucian, B., Dunne, P., Friedman, H., Ragsdale, R., Pross, S., Widen, R., 1996. Detection of altered T helper 1 and T helper 2 cytokine production by peripheral blood mononuclear cells in patients with multiple sclerosis utilizing intracellular cytokine detection by flow cytometry and surface marker analysis. Clin. Diagn. Lab. Immunol 3, 411.

de Almeida, A.B., Silva, M.C., Braga, C., Freedman, D.O., 1998. Differences in the frequency of cytokine-producing cells in antigenemic and nonantigenemic individuals with bancroftian filariasis. Infect. Immun. 66, 1377.

Dinter, A., Berger, E.G., 1998. Golgi-disturbing agents. Histochem. Cell Biol. 109, 571.

Estcourt, C., Rousseau, Y., Sadeghi, H.M., Thieblemont, N., Carreno, M.P., Weiss, L., Haeffner, C.N., 1997. Flow-cytometric assessment of in vivo cytokine-producing monocytes in HIV-infected patients. Clin. Immunol Immunopathol. 83, 60.

Falchetti, R., Di, F.P., Lanzilli, G., Gaziano, R., Casalinuovo, I.A., Palamara, A.T., Ravagnan, G., Garaci, E., 1998. Determination of cytokine co-expression in individual splenic $CD4^+$ and $CD8^+$ T cells from influenza virus-immune mice. Immunology 95, 346.

Fallon, P.G., Smith, P., Dunne, D.W., 1998. Type 1 and type 2 cytokine-producing mouse $CD4^+$ and $CD8^+$ T cells in acute *Schistosoma mansoni* infection. Eur. J. Immunol 28, 1408.

Ferry, B., Antrobus, P., Huzicka, I., Farrell, A., Lane, A., Chapel, H., 1997. Intracellular cytokine expression in whole blood preparations from normals and patients with atopic dermatitis. Clin. Exp. Immunol 110, 410.

Garcia, V.E., Sieling, P.A., Gong, J., Barnes, P.F., Uyemura, K., Tanaka, Y., Bloom, B.R., Morita, C.T., Modlin, R.L., 1997. Single-cell cytokine analysis of gamma delta T cell responses to nonpeptide mycobacterial antigens. J. Immunol. 159, 1328.

Gift, E.A., Park, H.J., Paradis, G.A., Demain, A.L., Weaver, J.C., 1996. FACS-based isolation of slowly growing cells: double encapsulation of yeast in gel microdrops. Nat. Biotechnol. 14, 884.

Gross, D.M., Steere, A.C., Huber, B.T., 1998. T helper 1 response is dominant and localised to the synovial fluid in patients with Lyme arthritis. J. Immunol. 160, 1022.

Hamann, D., Baars, P.A., Hooibrink, B., van, L.R., 1996. Heterogeneity of the human $CD4^+$ T-cell population: two distinct $CD4^+$ T-cell subsets characterized by coexpression of CD45RA and CD45RO isoforms. Blood 88, 3513.

Honda, J., Okubo, Y., Tanaka, K., Kusaba, M., Kumagai, M., Saruwatari, N., Oizumi, K., 1998. CD3-CD4-CD8-CD56-

CD19-CD14− cells and CD3+CD8 dull-positive cells produce IL-4 in AIDS patients. Br. J. Haematol. 101, 74.

Hoyle, C., Bangs, C.D., Chang, P., Kamel, O., Mehta, B., Negrin, R.S., 1998. Expansion of Philadelphia chromosome-negative CD3(+)CD56(+) cytotoxic cells from chronic myeloid leukemia patients: in vitro and in vivo efficacy in severe combined immunodeficiency disease mice. Blood 92, 3318.

Hussell, T., Openshaw, P.J.M., 1998. Intracellular interferon-gamma expression in natural killer cells precedes lung $CD8^+$ T cell recruitment during respiratory syncytial virus infection. J. Gen. Virol. 79, 2593.

Hussell, T., Spender, L.C., Georgiou, A., O'Garra, A., Openshaw, P.J.M., 1996. Th1 and Th2 cytokine induction in pulmonary T-cells during infection with respiratory syncytial virus. J. Gen. Virol. 77, 2447.

Hussell, T., Khan, U., Openshaw, P.J.M., 1997a. IL-12 treatment attenuates Th2 and B cell responses but does not improve vaccine-enhanced lung illness. J. Immunol. 159, 328.

Hussell, T., Baldwin, C.J., O'Garra, A., Openshaw, P.J.M., 1997b. $CD8^+$ T-cells control Th2-driven pathology during pulmonary respiratory syncytial virus infection. Eur. J. Immunol. 27, 3341.

Hussell, T., Georgiou, A., Sparer, T.E., Matthews, S., Pala, P., Openshaw, P.J.M., 1998. Host genetic determinants of vaccine-induced eosinophilia during respiratory syncytial virus infection. J. Immunol. 161, 6215.

Isaacs, D., Bangham, C.R.M., McMichael, A.J., 1987. Cell-mediated cytotoxic response to respiratory syncytial virus in infants with bronchiolitis. Lancet ii (8562), 769.

Isaacs, D., MacDonald, N.E., Bangham, C.R.M., McMichael, A.J., Higgins, P.G., Tyrrell, D.A.J., 1991. The specific cytotoxic T-cell response of adult volunteers to infection with respiratory syncytial virus. Immun. Infect. Dis. 1, 5.

Ito, M., Watanabe, M., Kamiya, H., Sakurai, M., 1997. Changes in intracellular cytokine levels in lymphocytes induced by measles virus. Clin. Immunol. Immunopathol. 83, 281.

Ito, M., Koide, W., Sakurai, M., 1998. Changes in intracellular cytokine levels in newborn and adult lymphocytes induced by HSV-1. J. Med. Virol. 56, 145.

James, K., Skibinski, G., Hoffman, P., 1996. A comparison of the performance in vitro of precision cut tissue slices and suspensions of human spleen with special reference to immunoglobulin and cytokine production. Hum. Antibodies Hybridomas 7, 138.

Jason, J., Archibald, L., McDonald, L.C., Hart, W.M., Rheanppumikankit, S., Tansuphwaswadikul, S., Byrd, M.G., Larned, J., Han, A., Green, T.A., Jarvis, W.R., 1999. Immune determinants of organism and outcome in febrile hospitalized Thai patients with bloodstream infections. Clin. Diagn. Lab. Immunol 6, 73.

Jason, J., Larned, J., 1997. Single-cell cytokine profiles in normal humans: comparison of flow cytometric reagents and stimulation protocols. J. Immunol. Methods 207, 13.

Jenne, L., Kilwinski, J., Radloff, P., Flick, W., Kern, P., 1998. Clinical efficacy of and immunologic alterations caused by interferon gamma therapy for alveolar echinococcosis. Clin. Infect. Dis. 26, 492.

Jung, T., Schauer, U., Heusser, C., Neumann, C., Rieger, C., 1993. Detection of intracellular cytokines by flow cytometry. J. Immunol. Methods 159, 197.

Jung, T., Lack, G., Schauer, U., Uberuck, W., Renz, H., Gelfand, E.W., Rieger, C.H., 1995. Decreased frequency of interferon-gamma- and interleukin-2-producing cells in patients with atopic diseases measured at the single cell level. J. Allergy Clin. Immunol. 96, 515.

Jung, T., Wijdenes, J., Neumann, C., de, V.J., Yssel, H., 1996. Interleukin-13 is produced by activated human $CD45RA^+$ and $CD45RO^+$ T cells: modulation by interleukin-4 and interleukin-12. Eur. J. Immunol 26, 571.

Kadena, T., Matsuzaki, G., Fujise, S., Kishihara, K., Takimoto, H., Sasaki, M., Beppu, M., Nakamura, S., Nomoto, K., 1997. TCR alpha beta$^+$ CD4− CD8− T cells differentiate extrathymically in an lck-independent manner and participate in early response against Listeria monocytogenes infection through interferon-gamma production. Immunology 91, 511.

Kelleher, P., Knight, S.C., 1998. IL-12 increases CD80 expression and the stimulatory capacity of bone marrow-derived dendritic cells. Int. Immunol 10, 749.

Knudsen, E., Seierstad, T., Vaage, J.T., Naper, C., Benestad, H.B., Rolstad, B., Maghazachi, A.A., 1997. Cloning, functional activities and in vivo tissue distribution of rat NKR-$P1^+$ TCR alpha beta$^+$ cells. Int. Immunol 9, 1043.

Kon, O.M., Sihra, B.S., Till, S.J., Corrigan, C.J., Kay, A.B., Grant, J.A., 1998. Unstimulated basophils in atopic and nonatopic subjects express intracellular interleukin-4: detection by flow cytometry. Allergy 53, 891.

Krouwels, F.H., Nocker, R.E., Snoek, M., Lutter, R., van-der, Z.J., Weller, F.R., Jansen, H.M., Out, T.A., 1997. Immunocytochemical and flow cytofluorimetric detection of intracellular IL-4, IL-5 and IFN-gamma: applications using blood- and airway-derived cells. J. Immunol. Methods 203, 89.

Krug, N., Schauer, U., Wagner, T.O., 1997. [Cell biology of bronchial asthma: interesting new methods. Intracellular determination of cytokines in T-cells and basic proteins in eosinophilic granulocytes using flow cytometry]. Pneumologie 51, 381.

Krug, N., Jung, T., Napp, U., Wagner, K., Schultze, W.G., Heusser, C., Rieger, C.H., Schauer, U., Fabel, H., 1998. Frequencies of T cells expressing interleukin-4 and interleukin-5 in atopic asthmatic children. Comparison with atopic asthmatic adults. Am. J. Respir. Crit. Care Med. 158, 754.

Kubota, R., Kawanishi, T., Matsubara, H., Manns, A., Jacobson, S., 1998. Demonstration of human T lymphotropic virus type I (HTLV-I. tax-specific $CD8^+$ lymphocytes directly in peripheral blood of HTLV-I-associated myelopathy/tropical spastic paraparesis patients by intracellular cytokine detection. J. Immunol. 161, 482.

Kusaba, M., Honda, J., Fukuda, T., Oizumi, K., 1998. Analysis of type 1 and type 2 T cells in synovial fluid and peripheral blood of patients with rheumatoid arthritis. J. Rheumatol. 25, 1466.

Labarriere, N., Pandolfino, M.C., Raingeard, D., Le, G.S., Diez, E., Le, D.E., Dreno, B., Jotereau, F., 1998. Frequency and relative fraction of tumor antigen-specific T cells among lymphocytes from melanoma-invaded lymph nodes. Int. J. Cancer 78, 209.

Lalvani, A., Brookes, R., Hambleton, S., Britton, W.J., Hill, A.V.,

McMichael, A.J., 1997. Rapid effector function in CD8$^+$ memory T cells. J. Exp. Med. 186, 859.

Lecoeur, H., Ledru, E., Gougeon, M.L., 1998. A cytofluorometric method for the simultaneous detection of both intracellular and surface antigens of apoptotic peripheral lymphocytes. J. Immunol. Methods 217, 11.

Lee, C.L., Lee, S.H., Jay, F.T., Rozee, K.R., 1990. Immunobiological study of interferon-gamma-producing cells after staphylococcal enterotoxin B stimulation. Immunology 70, 94.

Lohning, M., Grogan, J.L., Coyle, A.J., Yazdanbakhsh, M., Meisel, C., Gutierrez, R.J., Radbruch, A., Kamradt, T., 1999. T1/ST2 expression is enhanced on CD4$^+$ T cells from schistosome egg-induced granulomas: analysis of Th cell cytokine coexpression ex vivo. J. Immunol. 162, 3882.

Maino, V.C., Picker, L.J., 1998. Identification of functional subsets by flow cytometry: intracellular detection of cytokine expression. Cytometry 34, 207.

Manz, R., Assenmacher, M., Pfluger, E., Miltenyi, S., Radbruch, A., 1995. Analysis and sorting of live cells according to secreted molecules, relocated to a cell-surface affinity matrix. Proc. Natl. Acad. Sci. USA 92, 1921.

Meenan, J., Spaans, J., Grool, T.A., Lammers, K., Pals, S., Tytgat, G.N., van, D.S., 1998. Variation in gut-homing CD27-negative lymphocytes in inflammatory colon disease. Scand. J. Immunol. 48, 318.

Meyaard, L., Hovenkamp, E., Keet, I.P., Hooibrink, B., de, J.I., Otto, S.A., Miedema, F., 1996. Single cell analysis of IL-4 and IFN-gamma production by T cells from HIV-infected individuals: decreased IFN-gamma in the presence of preserved IL-4 production. J. Immunol. 157, 2712.

Miner, K.T., Croft, M., 1998. Generation, persistence, and modulation of Th0 effector cells: role of autocrine IL-4 and IFN-gamma. J. Immunol. 160, 5280.

Morita, Y., Yamamura, M., Kawashima, M., Harada, S., Tsuji, K., Shibuya, K., Maruyama, K., Makino, H., 1998. Flow cytometric single-cell analysis of cytokine production by CD4$^+$ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. Arthritis Rheum. 41, 1669.

Mosmann, T.R., Cherwinski, H.M., Bond, M.W., Giedlin, M.A., Coffman, R.L., 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. 136, 2348.

Murali, K.K., Altman, J.D., Suresh, M., Sourdive, D., Zajac, A., Ahmed, R., 1998. In vivo dynamics of anti-viral CD8 T cell responses to different epitopes. An evaluation of bystander activation in primary and secondary responses to viral infection. Adv. Exp. Med. Biol. 452, 123.

Murphy, E., Shibuya, K., Hosken, N.A., Openshaw, P.J.M., Manio, V., Davis, K., Murphy, K., O'Garra, A., 1996. Reversibility of T helper 1 and T helper 2 populations is lost after long term stimulation. J. Exp. Med. 183, 901.

Nakayama, K., Nakauchi, H., 1993. Cyclosporin A inhibits the decrease of CD4/CD8 expression induced by protein kinase C activation. Int. Immunol. 5, 419.

Nakazawa, M., Sugi, N., Kawaguchi, H., Ishii, N., Nakajima, H., Minami, M., 1997. Predominance of type 2 cytokine-producing CD4$^+$ and CD8$^+$ cells in patients with atopic dermatitis. J. Allergy Clin. Immunol 99, 673.

Nakagawa, S., Aiba, S., Tagami, H., 1998. Decreased frequency of interferon-gamma-producing CD4$^+$ cells in the peripheral blood of patients with atopic dermatitis. Exp. Dermatol. 7, 112.

Nazaruk, R.A., Rochford, R., Hobbs, M.V., Cannon, M.J., 1998. Functional diversity of the CD8(+) T-cell response to Epstein-Barr virus (EBV): implications for the pathogenesis of EBV-associated lymphoproliferative disorders. Blood 91, 3875.

Nir, R., Lamed, R., Gueta, L., Sahar, E., 1990. Single-cell entrapment and microcolony development within uniform microspheres amenable to flow cytometry. Appl. Environ. Microbiol. 56, 2870.

North, M.E., Ivory, K., Funauchi, M., Webster, A.D., Lane, A.C., Farrant, J., 1996. Intracellular cytokine production by human CD4$^+$ and CD8$^+$ T cells from normal and immunodeficient donors using directly conjugated anti-cytokine antibodies and three-colour flow cytometry. Clin. Exp. Immunol 105, 517.

North, M.E., Webster, A.D., Farrant, J., 1998. Primary defect in CD8$^+$ lymphocytes in the antibody deficiency disease (common variable immunodeficiency): abnormalities in intracellular production of interferon-gamma (IFN-gamma) in CD28$^+$ ('cytotoxic') and CD28$-$ ('suppressor') CD8$^+$ subsets. Clin. Exp. Immunol 111, 70.

O'Mahony, L., Holland, J., Jackson, J., Feighery, C., Hennessy, T.P., Mealy, K., 1998. Quantitative intracellular cytokine measurement: age-related changes in proinflammatory cytokine production. Clin. Exp. Immunol 113, 213.

Openshaw, P., Murphy, E.E., Hosken, N.A., Maino, V., Davis, K., Murphy, K., O'Garra, A., 1995. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. J. Exp. Med. 182, 1357.

Openshaw, P.J.M., Sparer, T.E., Spender, L., Matthews, S., Pala, P., Hussell, T., 1998. Investigative measures for airway inflammation: viruses and T-helper 2 responses. Eur. Respir. Rev. 8, 1128.

Oxenius, A., Karrer, U., Zinkernagel, R.M., Hengartner, H., 1999. IL-12 is not required for induction of type 1 cytokine responses in viral infections. J. Immunol. 162, 965.

Paglieroni, T.G., Perez, R., Katznelson, S., Muto, K., Chang, T., Scott, S., MacKenzie, M.R., Holland, P.V., 1999. Donor cell induced CD69 expression and intracellular IL-2 and IL-4 production by peripheral blood lymphocytes isolated from kidney transplant recipients. Hum. Immunol 60, 41.

Panichi, V., De, P.S., Andreini, B., Migliori, M., Tessore, V., Taccola, D., Rindi, P., Palla, R., Tetta, C., 1998. Cytokine production in haemodiafiltration: a multicentre study. Nephrol. Dial. Transplant. 13, 1737.

Pelchen, M.A., Parsons, I.J., Marsh, M., 1993. Phorbol ester-induced downregulation of CD4 is a multistep process involving dissociation from p56lck, increased association with clathrin-coated pits, and altered endosomal sorting. J. Exp. Med. 178, 1209.

Petersen, C.M., Christensen, E.I., Andresen, B.S., Moller, B.K., 1992. Internalisation, lysosomal degradation and new synthesis of surface membrane CD4 in phorbol ester-activated T-lymphocytes and U-937 cells. Exp. Cell Res. 201, 160.

Prussin, C., 1997. Cytokine flow cytometry: understanding cytokine biology at the single-cell level. J. Clin. Immunol. 17, 195.

Prussin, C., Metcalfe, D.D., 1995. Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. J. Immunol. Methods 188, 117.

Randolph, D.A., Carruthers, C.J., Szabo, S.J., Murphy, K.M., Chaplin, D.D., 1999. Modulation of airway inflammation by passive transfer of allergen-specific Th1 and Th2 cells in a mouse model of asthma. J. Immunol. 162, 2375.

Roura, M.C., Catalfamo, M., Sospedra, M., Alcalde, L., Pujol, B.R., Jaraquemada, D., 1997. Single-cell analysis of intrathyroidal lymphocytes shows differential cytokine expression in Hashimoto's and Graves' disease. Eur. J. Immunol 27, 3290.

Ruegg, C.L., Rajasekar, S., Stein, B.S., Engleman, E.G., 1992. Degradation of CD4 following phorbol-induced internalization in human T lymphocytes. Evidence for distinct endocytic routing of CD4 and CD3. J. Biol. Chem. 267, 18837.

Rumbley, C.A., Sugaya, H., Zekavat, S.A., El, R.M., Perrin, P.J., Phillips, S.M., 1999. Activated eosinophils are the major source of Th2-associated cytokines in the schistosome granuloma. J. Immunol. 162, 1003.

Sander, B., Andersson, J., Andersson, U., 1991. Assessment of cytokines by immunofluorescence and the paraformaldehyde-saponin procedure. Immunol. Rev. 119, 65.

Sander, B., Skansen, S.U., Damm, O., Hakansson, L., Andersson, J., Andersson, U., 1995. Sequential production of Th1 and Th2 cytokines in response to live bacillus Calmette-Guerin. Immunology 86, 512.

Sato, A., Tsuji, K., Yamamura, M., Morita, Y., Kanzaki, H., Tada, J., Makino, H., Arata, J., 1998a. Increased type 2 cytokine expression by both $CD4^+$ $CD45RO^+$ T cells and $CD8^+$ $CD45RO^+$ T cells in blood circulation is associated with high serum IgE but not with atopic dermatitis. J. Invest. Dermatol. 111, 1079.

Sato, M., Goto, S., Kaneko, R., Ito, M., Sato, S., Takeuchi, S., 1998b. Impaired production of Th1 cytokines and increased frequency of Th2 subsets in PBMC from advanced cancer patients. Anticancer Res. 18, 3951.

Schmitz, J., Assenmacher, M., Radbruch, A., 1993. Regulation of T helper cell cytokine expression: functional dichotomy of antigen-presenting cells. Eur. J. Immunol. 23, 191.

Sewell, W.A., North, M.E., Webster, A.D., Farrant, J., 1997. Determination of intracellular cytokines by flow-cytometry following whole-blood culture. J. Immunol. Methods 209, 67.

Simpson, S.J., Hollander, G.A., Mizoguchi, E., Allen, D., Bhan, A.K., Wang, B., Terhorst, C., 1997. Expression of pro-inflammatory cytokines by TCR alpha beta$^+$ and TCR gamma delta$^+$ T cells in an experimental model of colitis. Eur. J. Immunol 27, 17.

Skansen, S.U., Lindfors, A., Andersson, U., 1993. Cytokine production in mononuclear cells of human milk studied at the single-cell level. Pediatr. Res. 34, 213.

Skibinski, G., Hoffmann, P., Radbruch, A., James, K., 1997. Organ culture of human lymphoid tissue. II. Marked differences in cytokine production and proliferation between slice and suspension cultures of human spleen. J. Immunol. Methods 205, 115.

Smit, M.Z., Mattapallil, J.J., Villinger, F., Ansari, A.A., Dandekar, S., 1998. Intracellular cytokine expression in the $CD4^+$ and $CD8^+$ T cells from intestinal mucosa of simian immunodeficiency virus infected macaques. J. Med. Primatol. 27, 129.

Sommer, F., Faller, G., Konturek, P., Kirchner, T., Hahn, E.G., Zeus, J., Rollinghoff, M., Lohoff, M., 1998a. Antrum- and corpus mucosa-infiltrating CD4(+) lymphocytes in *Helicobacter pylori* gastritis display a Th1 phenotype. Infect. Immun. 66, 5543.

Sommer, F., Meixner, M., Mannherz, M., Ogilvie, A.L., Rollinghoff, M., Lohoff, M., 1998b. Analysis of cytokine patterns produced by individual $CD4^+$ lymph node cells during experimental murine leishmaniasis in resistant and susceptible mice. Int. Immunol 10, 1853.

Spender, L.C., Hussell, T., Openshaw, P.J., 1998. Abundant IFN-gamma production by local T cells in respiratory syncytial virus-induced eosinophilic lung disease. J. Gen. Virol. 79, 1751.

Stinn, J.L., Taylor, M.K., Becker, G., Nagano, H., Hasegawa, S., Furakawa, Y., Shimizu, K., Libby, P., Mitchell, R.N., 1998. Interferon-gamma-secreting T-cell populations in rejecting murine cardiac allografts: assessment by flow cytometry. Am. J. Pathol. 153, 1383.

Su, H.C., Cousens, L.P., Fast, L.D., Slifka, M.K., Bungiro, R.D., Ahmed, R., Biron, C.A., 1998. $CD4^+$ and $CD8^+$ T cell interactions in IFN-gamma and IL-4 responses to viral infections: requirements for IL-2. J. Immunol. 160, 5007.

Sugi, I.N., Nakazawa, M., Nakamura, S., Ohno, S., Minami, M., 1998. Increased frequencies of interleukin-2- and interferon-gamma-producing T cells in patients with active Behcet's disease. Invest. Ophthalmol. Vis. Sci. 39, 996.

Tabata, T., Hazama, S., Yoshino, S., Oka, M., 1999. Th2 subset dominance among peripheral blood T lymphocytes in patients with digestive cancers. Am. J. Surg. 177, 203.

Telerman, A., Amson, R.B., Romasco, F., Wybran, J., Galand, P., Mosselmans, R., 1987. Internalization of human T lymphocyte receptors. Eur. J. Immunol 17, 991.

Thoma, S., Bonhagen, K., Vestweber, D., Hamann, A., Reimann, J., 1998. Expression of selectin-binding epitopes and cytokines by $CD4^+$ T cells repopulating scid mice with colitis. Eur. J. Immunol 28, 1785.

Ulrich, P., Vohr, H.W., 1996. Murine lymph node antigen presenting cells are the main source of interleukin-6 in the initiation of delayed-type hypersensitivity. Eur. Cytokine Netw. 7, 401.

Westby, M., Marriott, J.B., Guckian, M., Cookson, S., Hay, P., Dalgleish, A.G., 1998. Abnormal intracellular IL-2 and interferon-gamma (IFN-gamma) production as HIV-1-associated markers of immune dysfunction. Clin. Exp. Immunol 111, 257.

Xu, D., Chan, W.L., Leung, B.P., Huang, F., Wheeler, R., Piedrafita, D., Robinson, J.H., Liew, F.Y., 1998. Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. J. Exp. Med. 187, 787.

Zunino, S.J., Singh, M.K., Bass, J., Picker, L.J., 1996. Immunodetection of histone epitopes correlates with early stages of apoptosis in activated human peripheral T lymphocytes. Am. J. Pathol. 149, 653.